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**Graphical abstract**

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Complement and inflammasome overactivation mediates paroxysmal nocturnal hemoglobinuria with autoinflammation

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Patients with paroxysmal nocturnal hemoglobinuria (PNH) have a clonal population of blood cells deficient in glycosylphosphatidylinositol-anchored (GPI-anchored) proteins, resulting from a mutation in the X-linked gene PIGA. Here we report on a set of patients in whom PNH results instead from biallelic mutation of PIGT on chromosome 20. These PIGT-PNH patients have clinically typical PNH, but they have in addition prominent autoinflammatory features, including recurrent attacks of aseptic meningitis. In all these patients we find a germ-line point mutation in one PIGT allele, whereas the other PIGT allele is removed by somatic deletion of a 20q region comprising maternally imprinted genes implicated in myeloproliferative syndromes. Unlike in PIGA-PNH cells, GPI is synthesized in PIGT-PNH cells and, since its attachment to proteins is blocked, free GPI is expressed on the cell surface. From studies of patients’ leukocytes and of PIGT-KO THP-1 cells we show that, through increased IL-1β secretion, activation of the lectin pathway of complement and generation of C5b-9 complexes, free GPI is the agent of autoinflammation. Eculizumab treatment abrogates not only intravascular hemolysis, but also autoinflammation. Thus, PIGT-PNH differs from PIGA-PNH both in the mechanism of clonal expansion and in clinical manifestations.

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematopoietic stem cell (HSC) disorder characterized by complement-mediated hemolysis, thrombosis, and bone marrow failure (1, 2). Affected cells harbor a somatic mutation in the PIGA gene, essential for the initial step in glycosylphosphatidylinositol (GPI) biosynthesis that occurs in the endoplasmic reticulum (ER) (Figure 1A, top and ref. 3). Loss of GPI biosynthesis results in the defective expression of GPI-anchored proteins (GPI-APs), including complement inhibitors CD59 and DAF/CD55 (Figure 1A, middle). The affected stem cells generate large numbers of abnormal blood cells after clonal expansion that occurs under bone marrow failure. The affected erythrocytes are defective in complement regulation and destroyed by the membrane attack complex (MAC or C5b-9) upon complement activation (1). Eculizumab, an anti-complement component 5 (C5) monoclonal antibody (mAb), has been used to prevent intravascular hemolysis and thrombosis (4, 5). Eculizumab binds to C5 and inhibits its activation and subsequent generation of C5b-9 complexes.

Among more than 20 genes involved in GPI biosynthesis and transfer to proteins, PIGA is X-linked whereas all others are autosomal (6). Because of X-linkage, one somatic mutation in PIGA causes GPI deficiency in both males and females (3). In contrast, 2 mutations are required for an autosomal gene, but the probability of somatic mutations in both alleles at the same locus is extremely low, which explains why GPI deficiency in most patients with PNH is caused by PIGA somatic mutations. Recently, we reported 2 patients with PNH whose GPI-AP deficiency was caused by germline and somatic mutations in the PIGT gene localized on chromosome 20q (7, 8). Both patients had a heterozygous germline loss-of-function mutation in PIGT, along with loss of the normal allele of PIGT by a deletion of 8 Mb or 18 Mb occurring in HSCs (7, 8). PIGT, forming a GPI transamidase complex with PIGK, PIGS, PIGU, and GPAA1, acts in the transfer of preassembled GPI to...
proteins in the ER (Figure 1A, top and ref. 9). In PIGT-defective cells, GPI is synthesized but is not transferred to precursor proteins, resulting in GPI-AP deficiency on the cell surface (Figure 1A, bottom). We showed recently that non–protein-linked, free GPI remaining in the ER of PIGT-defective Chinese hamster ovary (CHO) cells is transported to and displayed on the cell surface (Figure 1A, bottom and ref. 10).

Two reported PNH patients with PIGT defect suffered from recurrent inflammatory symptoms that are unusual in patients with PNH (7, 8). Here, we report 2 more patients with PNH who lost PIGT function via a similar genetic mechanism, and present insights into the expansion of PIGT-defective clones common among 4 patients. We also present the integrated clinical characteristics of these 4 patients and show that PIGT-defective mononuclear leukocytes, but not PIGA-defective mononuclear leukocytes, secreted IL-1β in response to inflammasome activators. Using a PIGT-KO THP-1 cell model, we show that complement activation is enhanced on the surface of PIGT-defective cells leading to MAC-dependent elevated secretion of IL-1β. Against this background, we propose a distinct disease entity, PIGT-PNH.

Results

Case report. Japanese patient J1 (8) and German patients G1 (7), G2, and G3 were diagnosed with PNH at the ages of 68, 49, 65, and 66, respectively. The changes in PNH clone sizes in J1, G1, and G3 after PNH diagnosis are shown in Figure 1B. They were treated with eculizumab, which effectively prevented intravascular hemolysis. Percentage of PNH cells rapidly increased after commencement of eculizumab, but not IL-1β in response to inflammasome activators. Using a PIGT-KO THP-1 cell model, we show that complement activation is enhanced on the surface of PIGT-defective cells leading to MAC-dependent elevated secretion of IL-1β. Against this background, we propose a distinct disease entity, PIGT-PNH.

The tumor suppressor-like gene L3MBTL1 and the kinase gene SGK2 located within the myeloid CDR (Figure 2, A and B) are expressed only in the paternal allele due to gene imprinting (16). It was shown that losses of active paternal alleles of these 2 genes had a causal relationship with clonal expansion of these 20q− myeloid cells (13). L3MBTL1 and SGK2 transcripts were undetectable in GPI-AP−defective granulocytes from J1 and extremely low in whole blood cells from G1, whereas they were found in granulocytes from healthy individuals (Figure 2C). The transcripts of 2 unimprinted genes, IFT52 and MYB, were detected in both GPI-AP−defective granulocytes from J1 and normal granulocytes (Figure 2C, top). The results therefore indicate that the expression of L3MBTL1 and SGK2 is lost in GPI-AP−defective cells in J1 and G1.

The results shown in Figure 2C also indicate that the somatically deleted region in J1 and G1 included active L3MBTL1 and SGK2, so it was in the paternal chromosome. Owing to mRNA from patients G2 and G3 not being available, we determined the methylation status of the L3MBTL1 gene using DNA from blood leukocytes, among which the large majority of cells were of the PNH phenotype. L3MBTL1 in G1 and G3 samples was hypermethylated (Figure 2D and Supplemental Figure 3A), indicating that the myeloid CDR allele remaining in their PNH clones was imprinted. In contrast, the G2 sample was hypomethylated. It was reported that, in some MPN patients with myeloid CDR deletion, the remaining allele was hypomethylated; nevertheless, its transcription was suppressed (13). G2 might be in a similar situation, although it was not possible to draw a definitive conclusion on this by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis as the patient had passed away. These results indicate that the loss of expressed myeloid CDR allele is associated with clonal expansion of PIGT-defective cells similar to 20q− MPN and MDS.

Appearance of free GPI on the surface of PIGT-defective cells. PIGA is required for the first step in GPI biosynthesis (17); therefore, no
Figure 1. Clinical features of PIGT-PNH. (A) Schematics of normal and defective biosynthesis of GPI-APs. (Top) In normal cells, GPI is synthesized in the ER from phosphatidylinositol (PI) by sequential reactions and assembled GPI is attached to proteins (orange oval). PIGA acts in the first step whereas PIGT acts in attachment of GPI to proteins. GPI-APs are transported to the plasma membrane (PM). (Middle) No GPI biosynthesis in PNH caused by PIGA defect. (Bottom) Accumulation of free GPI in PNH cells caused by PIGT defect. Non–protein-linked GPI is transported to the PM. (B) Time course of PNH clone sizes in patients G1, G3, and J1. Percentages of PNH cells in monocytes, granulocytes, erythrocytes, and reticulocytes are plotted as a function of time in days. Arrows, start of eculizumab therapy. (C) Examples of urticaria in G3 before the start of the anakinra treatment are shown on the left (chest) and middle (left upper leg); hemoglobinuria in G3 is shown on the right. Brightness was adjusted in the bottom chest image to more clearly show raised skin in the affected area. The pictures were made available by the patient. (D) Clinical courses of G3 in comparison to J1 (Figure 1 in ref. 8 was modified with additional data) including effective treatments. G3 (top) had meningitis 19 times between 62–65 years of age. Eculizumab therapy started at 66 years of age after a severe hemolysis. J1 (bottom) had meningitis 12 times between 53–69 years of age when eculizumab therapy started. Downward green arrows, onset of urticaria and/or arthralgia; blue middle height bars, meningitis; orange short bars, hemolysis; orange long bars, severe hemolysis; horizontal arrows of various lengths, treatment periods of effective therapies (anakinra and canakinumab were given with prednisolone); upward arrows with number and asterisk, serum samples taken for cytokine and other protein determination.
Table 1. Summary of clinical and genetic findings

<table>
<thead>
<tr>
<th></th>
<th>J1*</th>
<th>G1*</th>
<th>G2*</th>
<th>G3*</th>
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<tr>
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<tr>
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<table>
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<th>Signs and symptoms (age of onset)</th>
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<th>G1*</th>
<th>G2*</th>
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<tbody>
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<td>Urticaria</td>
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<td>+ (26)</td>
<td>+ (48)</td>
</tr>
<tr>
<td>Arthralgia</td>
<td>+ (30)²</td>
<td>+ (27)</td>
<td>+ (61)</td>
</tr>
<tr>
<td>Myalgia</td>
<td>+ (27)²</td>
<td>+ (27)²</td>
<td>+ (61)²</td>
</tr>
<tr>
<td>Aseptic meningitis</td>
<td>+ (53)²</td>
<td>+ (53)²</td>
<td>+ (61)²</td>
</tr>
<tr>
<td>Fever</td>
<td>+ (53)²</td>
<td>+ (53)²</td>
<td>+ (61)²</td>
</tr>
<tr>
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<td>+ (44)²</td>
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<td>Abdominal pain</td>
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<td>Ulcerative colitis</td>
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<th><strong>Previous therapy</strong></th>
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<th>Corticosteroids, diphenhydramine, cromoglycin, azathioprine (for symptoms of autoinflammation)</th>
<th>Corticosteroids (for residual hemolysis after start of eculizumab)</th>
<th>Corticosteroids, mycophenolate mofetil, dapsone, anakinra, canakinumab (for symptoms of autoinflammation)</th>
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<td>+ (69)²</td>
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<td>P allele of PIGT²</td>
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<td>Entire deletion (8 Mb)</td>
<td>Entire deletion (12 Mb)</td>
<td>Entire deletion (15 Mb)</td>
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*J1 had recurrent urticaria and arthralgia since 30 years of age. At 53 years of age, he had first aseptic meningitis and since then had meningitis several times a year. A cerebrospinal fluid sample taken during an episode of meningitis had approximately 2000 polymorphonuclear leukocytes per microliter. Since 58 years of age, frequencies of meningitis increased to twice a month. At 67 years of age, intravascular hemolysis was first noted. His white blood cell counts during the asymptomatic period and after commencement of eculizumab therapy were within a normal range. Refer to ref. 8 for more detailed description of J1. ²Refer to the Supplemental Data in ref. 7 for detailed description of G1. ³In G2, 96% of granulocytes and 19% of erythrocytes were GPI-AP–defective. Additional diagnoses and symptoms for G2: arteriosclerosis with coronary heart disease, myocardial infarction (49 years), recurrent left brain transient ischemic attacks (66 years), recurrent confusion symptoms and disturbance of memory; cholecystectomy (43 years) in the years following remission of cholangitis; elevation of indirect bilirubin; 8 chronic lymphocyctic leukemia (64 years), treatment with rituximab (whether leukemic cells were from PNH clone was not known); hemochromatosis (compound heterozygous), diagnosed at age of 66 because of elevation of ferritin, prostate adenoma. The patient died for cardiac decompensation with fulminant pulmonal edema and massive general arteriosclerosis with ulcerated plaques. Additional diagnosis and symptoms for G3: cholecystolithiasis (65 years); metabolic syndrome with obesity, diabetes, and dyslipoproteinemia; sensorineural hearing loss (61 years); peripheral arterial occlusive disease; JAK2 V617F mutation (age of 66) (whether the mutation occurred in PNH clone was not known), neurinoma left side (52 years), increasing personality changes, and disturbance of memory since the beginning of the meningitis episodes until start of eculizumab. Plus signs indicate presence of corresponding signs and symptoms. Numbers in parentheses indicate age when the symptom was first recognized or eculizumab treatment was started. ⁴Headache often occurred associated with urticaria (first urticaria, followed by severe headache). ⁵First time of significantly elevated lactate dehydrogenase (LDH) (7). ⁶Patient reported dark urine (Figure 1C, right). G1 developed ulcerative colitis/proctitis about 5 years after diagnosis of PNH. Eculizumab was not effective to ulcerative colitis/proctitis in G1. ⁷M, maternal; P, paternal. Based on NM_015937. ⁸The same variant c.250G > T in J1 (8) was found in 2 Japanese patients with inherited GPI deficiency (IGD) suffering from developmental delay, seizures, and skeletal abnormality (Supplemental Table 2 and refs. 11, 37). ⁹Data are shown in Supplemental Figure 2. ²Deletions of J1 and G2 included entire PIGU gene as well as PIGT.

GPI intermediate is generated in PIGA-defective cells (Figure 1A, middle). PIGT is involved in the attachment of GPI to proteins. GPI is synthesized in the ER, but is not used as a protein anchor in PIGT-defective cells (Figure 1A, bottom). We used T5 mAb that recognizes free GPI, but not protein-bound GPI, as a probe to characterize free GPI (Figure 3A and refs. 18, 19; see Methods for epitope and other characteristics of this antibody). Using T5 mAb in Western blotting and flow cytometry, we first compared PIGT-defective CHO cells with PIGL-defective CHO cells, in which an early GPI biosynthetic step is defective, like in PIGA-defective cells. T5 mAb revealed a strong band of free GPI at a position corresponding to approximately 10 kDa in lysates of PIGT-defective cells but not of PIGL-defective cells (Figure 3B). DAF and CD59 were not detected in either mutant cell, confirming that non-GPI-anchored precursor proteins were degraded (Figure 3B and ref. 20). T5 mAb stained the surface of PIGT-defective CHO cells but not PIGL-defective cells, confirming that free GPI transported to the cell surface is detectable by T5 mAb (Figure 3C).

We then analyzed blood cells from J1, G1, and G3, and from patients with PIGA-PNH by flow cytometry. All 4 patients had PNH-type blood cells defective in various GPI-APs (Figure 3, D–F, and Supplemental Figure 4, A and B). Erythrocytes from J1, G1, and G3 contained 3%, 84%, and 60% PNH cells, respectively, and a sizable fraction of them (36%, 87%, and 87%, respectively) were stained by T5 mAb (Figure 3D). J1 had PNH cells in granulocytes (81%), monocytes (87%), and B lymphocytes (54%), but not T lymphocytes (<2%), as revealed by anti-CD59 and GPI-binding probe fluorescence-labeled nonlytic aerolysin (FLAER) (21). Affected monocytes and B lymphocytes were strongly stained by T5 mAb, whereas affected granulocytes were weakly but clearly stained (Figure 3E).
stain PIGT-PNH cells. Aerolysin specifically binds to the GPI moiety of some but not all GPI-APs, and requires simultaneous association with N-glycan for high-affinity binding (25–27). Our results indicate that FLAER binds to protein-bound GPI but not to free GPI.

We next investigated whether GPI-AP–defective clone was present in the stage only with autoinflammation. After determining the break points causing the deletion of 18 Mb in J1 (Supplemental Figure 5), we quantitatively analyzed blood DNA samples for the presence of the break. It was estimated that a relative level of the break in the sample taken in a stage with autoinflammation only (#1) was approximately 10% of a level in the sample taken 1 month after start of eculizumab therapy (#2), which contained 29% PNH cells (Figure 3G). Therefore, approximately 3% of total leukocytes obtained 4 months before the onset of recurrent hemolysis were GPI-AP–defective cells.

and Supplemental Figure 4B). Normal populations in granulocytes, monocytes, and B lymphocytes were not stained by T5 mAb (Figure 3E). Similar results, showing strong T5 staining of affected monocytes and granulocytes, were obtained with leukocytes from G1 and G3 (Figure 3F). In contrast, PNH cells from PIGA-PNH patients and cells from healthy individuals were not positively stained by T5 mAb (Figure 3, E and F). Small fractions of WT erythrocytes from J1, G1, and G3 (0.13%, 9.7%, and 9.5%, respectively) were positively stained by T5 mAb (Figure 3D). Free GPI might be transferred from PNH cells to WT erythrocytes in vivo (22, 23), although the exact mechanism involved needs to be clarified. Thus, the surface expression of the T5 mAb epitope is specific for PIGT-defective cells and T5 mAb is useful to diagnose PIGT-PNH. It was noted that FLAER, which is conveniently used to stain cell-surface GPI-APs and to determine the affected cells in patients with PNH (21, 24), did not stain PIGT-PNH cells. Aerolysin specifically binds to the GPI moiety of some but not all GPI-APs, and requires simultaneous association with N-glycan for high-affinity binding (25–27). Our results indicate that FLAER binds to protein-bound GPI but not to free GPI.

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Figure 3. Biochemical abnormalities in PIGT-defective cells. (A) Schematic representation of binding specificity of T5 mAb. T5 mAb recognizes mammalian free GPI bearing GalNAc-side chain linked to the first mannose (left). T5 mAb does not bind to free GPI when Gal is attached to GalNAc (right). Man, mannose; GlcN, glucosamine; EtN-phosphate; PI, phosphatidylinositol. (B) Western blotting analysis of PIGT-defective and GIGL-defective CHO cells with T5 mAb for free GPI, anti-CD59, and anti-DAF mAbs, and anti-transferrin receptor (TR) as loading controls. (C) Flow cytometry of PIGT-defective and GIGL-defective CHO cells with T5 mAb and anti-CD59 mAb. (D) Flow cytometry of erythrocytes from G1, G1, G3, a healthy individual, and 2 patients with PIGA-PNH with T5 mAb and anti-CD59 (top panels) or anti-CD58 (bottom panels). (E) Flow cytometry of blood cells from G1, a healthy individual, and a patient with PIGA-PNH with T5 mAb and FLAER. (F) Granulocytes and monocytes from G1 and G3, a healthy individual, and a patient with PIGA-PNH, stained by T5 mAb and anti-CD59 mAb and anti-CD58 mAb. (G) Determination of the PNH clone size in J1 by qPCR analysis of the break causing 18 Mbp deletion. (Left) Threshold cycle in PCR for the break and exon 3 of PIGL as a reference. #1, DNA from whole blood leukocytes taken in a stage with autoinflammation only (4 months before the onset of recurrent hemolysis); #2, DNA from granulocytes (29% of cells were GPI-AP-defective) taken 1 month after start of eculizumab therapy. (Right) Relative levels of the break in samples #1 and #2 by setting the level in #2 as 1. Data are shown in mean of triplicate samples in 1 experiment. Mean RQ max values for #1 and #2 samples were 0.092 and 1.11, respectively.

Inflammasome- and complement-mediated autoinflammation, a feature of PIGT-PNH. IL-18 levels were elevated in serum samples taken from J1 before and after the commencement of eculizumab therapy (Table 2), suggesting that this phenomenon was not dependent upon C5 activation. Serum amyloid A was also elevated before eculizumab therapy, but was within the normal range after the commencement of eculizumab therapy (Table 2), suggesting that the elevation was dependent upon C5 activation. In G3, increased levels of soluble IL-2 receptor and thymidine kinase before, but not after, the start of eculizumab therapy suggested autoinflammation only (4 months before the onset of recurrent hemolysis); #2, DNA from whole blood leukocytes taken in a stage with autoinflammation only (4 months before the onset of recurrent hemolysis); #2, DNA from granulocytes (29% of cells were GPI-AP-defective) taken 1 month after start of eculizumab therapy. (Right) Relative levels of the break in samples #1 and #2 by setting the level in #2 as 1. Data are shown in mean of triplicate samples in 1 experiment. Mean RQ max values for #1 and #2 samples were 0.092 and 1.11, respectively.

To investigate the roles of complement in inflammasome activation in PIGT-PNH, we switched to a model cell system because patients’ blood cells were easily damaged in vitro under conditions of complement activation. PIGT-KO and PIGA-KO cells were generated from human monocytes THP-1 cells (Supplemental Figure 6A) and were differentiated to macrophages. They showed IL-1β response comparable to those of authentic inflammasome activators (Supplemental Figure 6B). To analyze the inflammasome response to activated complement, these THP-1-derived macrophages were stimulated with acidified serum (AS), which causes activation of the alternative complement pathway. PIGT-KO and PIGA-KO cells but not WT cells secreted IL-1β (1221.8 ± 91.6 pg/mL, 568.2 ± 101 pg/mL, and 237.2 ± 2.2 pg/mL for PIGT-KO, PIGA-KO, and WT cells, respectively) (Figure 5A). This result is consistent with impaired complement regulatory activities on PIGT-KO and PIGA-KO cells, and normal complement regulatory activity on WT cells. PIGT-KO cells secreted approximately twice as much IL-1β as PIGA-KO cells (P < 0.01). However, IL-1β production in G3 returned to normal levels after the transfection of PIGT and PIGA DNAs into PIGT-KO and PIGA-KO cells, respectively (Figure 5B). The levels of IL-1β mRNA and protein were comparable in WT, PIGT-KO, and PIGA-KO cells (Supplemental Figure 7A and B). Therefore, knock out of PIGT enhanced the secretion but not the generation of IL-1β.

Heat inactivation of complement and the addition of anti-C5 mAb to AS almost completely inhibited IL-1β secretion (Figure 5A). These results indicate that IL-1β secretion requires the activation of C5 on PIGT-KO and PIGA-KO cells. The activation of C5 leads to 2 biologically active products, C5a and MAC (29). To address which of these is important for IL-1β secretion, cells were treated with the C5aR antagonist W-54101 (30) or anti-C5aR mAb to inhibit the signal transduction through C5aR. WT, PIGT-KO, and PIGA-KO cells expressed C5aR at similar levels (Supplemental Figure 7C). The 2 methods of functional inhibition of C5aR had little effect on IL-1β secretion, indicating that the signal through C5aR plays no major role in this cell system (Figure 5C). Next, AS-treated cells were analyzed for surface binding of C3b fragments and MAC. Exposure to AS resulted in the higher binding of C3b fragments and MAC on PIGT-KO cells compared with that on PIGA-KO cells (Figure 5D and Supplemental Figure 8, A and B). The level of MAC was several times higher on PIGT-KO cells than on PIGA-KO cells, suggesting that complement activation was enhanced, leading to the enhanced formation of MAC on PIGT-KO cells. To confirm the role of MAC in IL-1β secretion, PIGT-KO cells were treated with acidified C6- and C7-depleted sera, in which C5a generation is intact whereas MAC formation is impaired. IL-1β secretion was greatly reduced by C6 or C7 depletion and was restored by the replenishment of C6 or C7 (Figure 5E). These results suggest that MAC but not C5a plays a critical role in IL-1β secretion.
role in the secretion of IL-1β. It is also suggested that free GPI plays some role in complement activation, leading to the enhanced binding of C3b fragments and MAC formation.

To determine whether the structure of free GPI (presence or absence of Gal capping) affects complement activation and subsequent IL-1β secretion, we knocked out SLC35A2 in PIGT-KO THP-1 cells. PIGT-SLC35A2 double-KO THP-1 cells were strongly stained by T5 mAb as expected (Supplemental Figure 6C). The binding of both C3b fragments and MAC increased approximately 5 times after SLC35A2 KO (Figure 5F). Concomitantly, the secretion of IL-1β more than doubled (Figure 5G). These results indicate that the structure of free GPI influenced complement activation efficiency and subsequent IL-1β secretion.

Finally, we asked whether only the alternative pathway is involved in binding of C3b fragments on PIGT-KO and PIGA-KO THP-1 cells, or the lectin and/or the classical pathway is also involved. For this, cells were stained with anti-C4d mAb after treatment with acidified serum because activation of either pathway would result in binding of C4b, which is in turn converted to C4d (31, 32). C4d fragments were bound on PIGT-KO cells and the binding was inhibited by 100 mM mannose by approximately 50% but not by N-acetylglucosamine (Figure 5H, bottom). These results suggest that the lectin pathway was activated on PIGT-KO THP-1 cells, accounting for at least 60% of C3b fragments. C4d fragments were bound on PIGA-KO cells at lower levels (60%–70% of PIGT-KO cell levels), which were not inhibited by either N-acetylglucosamine or mannose. Mannose, but not N-acetylglucosamine, mildly inhibited binding of C3b fragments on PIGA-KO cells (Figure 5H). It is unclear at the moment whether the classical pathway was involved in residual C4d binding on PIGT-KO cells in the presence of 100 mM mannose or in C4d binding on PIGA-KO cells.

**Discussion**

We studied patients with PNH caused by PIGT mutations. Because PNH caused by PIGT mutations is characterized by auto-inflammatory symptoms, we propose that they represent a new disease entity, PIGT-PNH. Patients with PIGT-PNH had a germline heterozygous mutation in the PIGT gene in combination with somatic deletion of the normal PIGT gene. Germline PIGT mutations were reported in patients with inherited GPI deficiency (IGD) (Supplemental Table 2), which is characterized by developmental delay, seizures, hypotonia, and typical facial dysmorphism (11, 33–39). Inflammatory symptoms and intravascular hemolysis were not reported in IGD patients with PIGT mutations. They had either partial loss-of-function homozygous mutations, or combinations of a partial loss-of-function mutation and a null or nearly null mutation. Therefore, cells from the patients with IGD have only partially reduced PIGT activities and express only partially reduced levels of CD59 and DAF/CD55, and may have free GPI only to a small extent. In contrast, both germline and somatic mutants in PIGT-PNH were functionally null or nearly null (Table 1). Therefore, the affected cells from PIGT-PNH patients lost CD59 and DAF/CD55 severely or completely and had high levels of free GPI. Interestingly, the same mutation c.250G>T (p.E84X) was found in J1 and 2 Japanese patients with IGD (11, 37) who were not related to each other. PIGT-PNH patient J1 and the mothers of 2 IGD patients from families 2 and 7 had the same heterozygous mutation (Table 1 and Supplemental Table 2). Therefore, the affected cells from PIGT-PNH patients lost CD59 and DAF/CD55 severely or completely and had high levels of free GPI. Interestingly, the same mutation c.250G>T (p.E84X) was found in J1 and 2 Japanese patients with IGD (11, 37) who were not related to each other. PIGT-PNH patient J1 and the mothers of 2 IGD patients from families 2 and 7 had the same heterozygous nonsense PIGT mutation (Table 1 and Supplemental Table 2). These mothers were healthy and no inflammatory symptoms were reported for them (11, 37), suggesting that autoinflammation of J1 was not caused by haploinsufficiency of PIGT but was initiat-

### Table 2. J1: cytokines and other proteins in serum samples

<table>
<thead>
<tr>
<th>Serum samples&lt;sup&gt;a&lt;/sup&gt;</th>
<th>*1, meningitis</th>
<th>*2, meningitis</th>
<th>*3, hemolysis</th>
<th>*4, 2.5 years after start of eculizumab</th>
<th>*5, 3.5 years after start of eculizumab</th>
<th>*6, 4.5 years after start of eculizumab</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-18 (pg/mL)</td>
<td>1410</td>
<td>797</td>
<td>972</td>
<td>1150</td>
<td>&lt;211.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>0.291</td>
<td>&lt;0.125</td>
<td>4.670</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>&lt;0.928</td>
</tr>
<tr>
<td>IL-1RA (pg/mL)</td>
<td>716</td>
<td>323</td>
<td>27500</td>
<td>239</td>
<td>85.6–660</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>127.0</td>
<td>239</td>
<td>2.30</td>
<td>6.32</td>
<td>2.94</td>
<td>6.32</td>
<td>2.410</td>
</tr>
<tr>
<td>Serum amyloid A (μg/mL)</td>
<td>17.5</td>
<td>2433.0</td>
<td>282.6</td>
<td>&lt;2.5</td>
<td>&lt;2.5</td>
<td>&lt;2.5</td>
<td>&lt;8.0</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>291</td>
<td>291</td>
<td>157</td>
<td>165</td>
<td>169</td>
<td>120–250</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Serum samples correspond to asterisked numerals in Figure 1D, representing samples from patient J1 during the indicated health events.

<table>
<thead>
<tr>
<th>Serum samples&lt;sup&gt;a&lt;/sup&gt;</th>
<th>*1, meningitis</th>
<th>*2, meningitis</th>
<th>*3, meningitis</th>
<th>*4, meningitis</th>
<th>*5, six months after start of eculizumab</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine kinase (U/L)</td>
<td>101</td>
<td>33.6</td>
<td>43.8</td>
<td>Not done</td>
<td>Normal</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Soluble IL-2 receptor (U/mL)</td>
<td>1750</td>
<td>1345</td>
<td>3026</td>
<td>1237</td>
<td>Normal</td>
<td>158–635</td>
</tr>
</tbody>
</table>

<sup>a</sup>Serum samples correspond to asterisked numerals in Figure 1D, representing samples from patient G3 during the indicated health events.
Japanese may have this variant (40). It is, therefore, highly likely that J1 inherited the PIGT variant.

The expansion of PIGA-defective clones in PNH is caused by selective survival under autoimmune bone marrow failure in most cases (41–43). This mechanism was first formulated by Rotoli and Luzzatto in 1989 (41). In few cases, clonal PNH cells acquired benign tumor characteristics by additional somatic mutations (44–46). In contrast, none of the patients with PIGT-PNH had documented bone marrow failure (Supplemental Figure 1A and refs. 7, 8). In addition, the myeloid CDR is lost in PIGT-defective clones in PIGT-PNH, similar to the case in clonal cells in myeloproliferative 20q- syndromes. The causal relationship between the myeloid CDR loss in PIGT-PNH and clonal expansion needs to be proven, particularly because boosted lineages under L3MBTL1 and SGK2 loss differ

Table 4. PIGA-PNH: cytokines and other proteins in serum samples

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-18 (pg/mL)</td>
<td>288</td>
<td>289</td>
<td>263</td>
<td>443</td>
<td>&lt;211.0</td>
</tr>
<tr>
<td>Serum amyloid A (μg/mL)</td>
<td>5.0</td>
<td>7.1</td>
<td>6.8</td>
<td>8.3</td>
<td>&lt;8.0</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>633</td>
<td>1190</td>
<td>923</td>
<td>1216</td>
<td>103–229</td>
</tr>
</tbody>
</table>

Data for 4 patients without eculizumab therapy.

Figure 4. IL-1β secretion from PIGT-PNH and PIGA-PNH cells. (A) The peripheral blood mononuclear cells from J1, PIGA-PNH4, and a healthy individual were incubated with 10–100 ng/mL Pam3CSK4 (Pam3) at 37°C for 4 hours, and then were incubated with 1.5 mM ATP for 30 minutes. IL-1β in the supernatants was measured by ELISA (top) and Western blotting (bottom). (Left) J1 (red bars) and a healthy individual (blue bars). (Right) PIGA-PNH4 (green bars) and a healthy individual (blue bars). P10, P50, and P100, 10, 50, and 100 ng/mL Pam3CSK4, respectively; H, healthy donors; T, PIGT-PNH; A, PIGA-PNH. (B) The peripheral blood mononuclear cells from J1, 2 or 3 patients with PIGA-PNH, and 2 or 5 healthy controls were stimulated with 200 ng/mL of Pam3 or 1 μg/mL of lipoteichoic acid (LTA) from Staphylococcus aureus for 4 hours at 37°C, and after washing were incubated with 3 mM ATP or 200 μg/mL monosodium urate (MSU) for 4 hours at 37°C. IL-1β secreted into the medium was determined by ELISA. Data for healthy donors and PIGA-PNH were shown as mean ± SD. Cells from 3 patients with PIGA-PNH (PIGA-PNH4–6) secreted very low levels of IL-1β (403 ± 326 pg/mL) after stimulation of NLRP3-inflammasomes with Pam3CSK4 and ATP under these strong conditions. In contrast, cells from PIGT-PNH J1 secreted IL-1β at a high level (25,000 pg/mL), a level that is higher than those from 3 healthy donors (6693 ± 1711 pg/mL). Similar results were obtained by stimulation with Pam3CSK4 and MSU instead of ATP. Moreover, similar results were obtained by stimulation with LTA plus ATP or MSU.
between in vitro study (13) and PIGT-PNH patients, and because platelet and leukocyte counts were not much changed in PIGT-PNH patients (Supplemental Figure 1A). Complement dysregulation, which is present in PIGT-PNH but not in myeloproliferative 20q- syndromes, might modulate blood cell profiles. Nevertheless, this unique deletion occurs in PIGT-PNH but not in PIGA-PNH. Taking these findings together, it is likely that the mechanism of clonal expansion for PIGT-PNH is distinct from that for PIGA-PNH cells (see models in Supplemental Figure 3B).

Whereas PIGT-PNH shares intravascular hemolysis and thrombosis with PNH, PIGT-PNH is characterized by autoinflammatory symptoms including recurrent urticaria, arthralgia, and aseptic meningitis. PIGT-PNH first manifested with autoinflammatory symptoms alone and symptoms of PNH became apparent many years later (Table 1). It is possible that different clinical symptoms appear depending on the size of the PIGT-defective clone. When the clone size is small, autoinflammation but not PNH may occur and, when the clone size becomes sufficiently large, PNH may become apparent. The idea that the PIGT-defective clone is small when only autoinflammation is seen was supported by analyzing J1 DNA obtained before the start of recurrent hemolysis, only approximately 3% of total leukocytes being PIGT-defective (Figure 3G). Inflammatory symptoms, recurrent urticaria, arthralgia, fever, and especially meningitis seen in PIGT-PNH are shared by children with cryopyrinopathies or cryopyrin-associated periodic syndrome (reviewed by Neven et al, ref. 47). Cryopyrinopathies are caused by gain-of-function mutation in NLRP3 that leads to easy activation of NLRP3 inflammasomes in monocytes and autoinflammatory symptoms (47), further suggesting that inflammasomes are activated in monocytes from PIGT-PNH patients. It was reported that autoinflammation occurs in patients having mosaicism with NLRP3-mutant cells even when the mutant clone size is small (frequency of mutant allele in whole-blood cells being 4.3% to 6.5%) (48). This is relevant to the symptoms/clone size relationship in PIGT-PNH as discussed above.

C5 activation must be involved in the autoinflammatory symptoms in PIGT-PNH because they were suppressed by eculizumab. It is important to consider GPI-AP deficiency for patients with recurrent autoinflammatory symptoms such as aseptic meningitis even when PNH symptoms are absent because eculizumab may be effective for such cases. Because DAF and CD59 are missing on PIGT-defective monocytes, C5a and MAC might be generated once complement activation has been initiated. It was reported that subarachnoidal application of C5a in rabbits and rats induced acute experimental meningitis (49). Various types of myeloid cells are present in the central nervous system (reviewed in ref. 50). If C5 activation occurs on some of those cells lacking complement regulatory function and C5a is generated, aseptic meningitis might ensue.

The involvement of complement in inflammasome activation has been shown in various blood cell systems (51-54). Indeed, in the THP-1 cell model system, IL-1β secretion was induced by complement in both PIGT-KO and PIGA-KO cells and more strongly in PIGT-KO cells, mainly through MAC formation (Figure 5, A and B). PIGT-PNH mononuclear cells were activated by conventional stimulators of inflammasomes similar to or even stronger than healthy control cells. Because blood mononuclear cells were easily lysed by acidified serum, the effect of complement on inflammasome activation in mononuclear cells could not be addressed. Taking the obtained findings together with the results for THP-1 cells, we speculate that PIGT-deficient monocytes show an enhanced inflammasome response when complement is activated. How free GPI is involved in inflammasome and complement activation needs to be further clarified to fully understand the mechanistic basis of PIGT-PNH.

PIGU is an essential component of GPI transamidase, forming a protein complex with PIGT, PIGS, PIGK, and GPAA1 (Figure 1A and ref. 55). PIGU-defective cells do not express GPI-APs on their surface (55). The PIGU gene is localized at approximately 7.4 Mb centromeric to the myeloid CDR (Figure 2, A and B), and regions of somatic deletions of 18 Mb and 12 Mb in GPI-AP-defective cells from J1 and G2, respectively, included the entire PIGU gene as well as myeloid CDR and PIGT gene. The levels of PIGU protein in these cells would be around half of the normal levels. It appears unlikely that the 50% reduction in PIGU has a significant impact on these cells. The levels of PIGT protein in the same cells would be zero or very low because mutations in the remaining PIGT gene are a nonsense mutation (E84X) in J1 and a frameshift mutation (frameshift after G254) in G2. For any remaining PIGT protein, half of the normal amounts of PIGU protein would be excessive for making the GPI transamidase complex. However, it is conceivable that, if a similar somatic deletion including PIGU and myeloid CDR occurs in a hematopoietic stem cell of an individual who bears a germline PIGU loss-of-function mutation, PNH with autoinflammation caused by PIGU mutation might occur.
Eculizumab was used for patients with PIGT-PNH. There are similarities and differences between PIGT-PNH and PNH against treatments with eculizumab. Similar to PNH, eculizumab was effective in preventing intravascular hemolysis in PIGT-PNH. Also similar to PNH, eculizumab caused appearance of direct Coombs-positive erythrocytes in patient G1, suggesting an induction of extravascular hemolysis (7). Patient G2 had residual hemolysis after start of eculizumab therapy, also suggesting extravascular hemolysis (Table 1). PIGT-KO THP-1 cells accumulated higher levels of C3b fragments upon complement activation (Figure 5D) and the affected erythrocytes from PIGT-PNH patients express free GPI (Figure 3D). Whether the free GPI-bearing PNH erythrocytes from PIGT-PNH patients accumulate more C3b fragments than PNH erythrocytes from PIGA-PNH patients and hence are more prone to extravascular hemolysis is yet to be clarified.

A difference is that percentage of PIGT-PNH cells increased not only in erythrocytes but also in granulocytes after treatment with eculizumab (Figure 1B). It is well known that percentage of PNH erythrocytes increases after eculizumab treatment because intravascular destruction of PNH erythrocytes is prevented by eculizumab (4). However, a sharp increase in percentage of PNH granulocytes seen in G1, G3, and J1 has not been reported in PIGA-PNH (4). A possible explanation for the difference is that PIGT-PNH granulocytes might have been destroyed in the blood by complement-dependent mechanisms. MAC formation was much higher on PIGT-KO THP-1 cells than on PIGA-KO cells (Figure 5D). If levels of MAC formation on PIGT-defective granulocytes are similarly high, MAC-mediated cell lysis might occur and eculizumab might prevent it, leading to an increase in the percentage of PNH granulocytes. Another possibility is that the commencement of eculizumab treatment was only coincident with the active phase of clonal expansion. Because hemolysis becomes apparent only when clone size has increased, it is still possible that use of eculizumab is not causally related to increase of PNH granulocytes but that eculizumab treatment was done during active clonal expansion.

Eculizumab was effective in preventing recurrent autoinflammatory symptoms of PIGT-PNH. After start of eculizumab therapy, aseptic meningitis has not occurred in patients G3 and J1, and other symptoms such as urticaria and arthralgia were also prevented (Figure 1D and ref. 8). These autoinflammatory symptoms, therefore, are dependent upon C5 activation. Results with PIGT-KO THP-1 cells indicated that IL-1β secretion after complement activation was mediated by MAC rather than C5a (Figure 5, C and E). Patient J1 had elevated levels of IL-18 and serum amyloid A (Table 2). After start of eculizumab, serum amyloid A level turned normal whereas IL-18 levels remained 4–5 times of the upper limit of normal. IL-18 secretion in PIGT-PNH, therefore, is not dependent upon terminal complement activation. C3 activation on the surface of PIGT-defective cells was higher than that on PIGA-defective cells, as suggested by experimental results with PIGT-KO and PIGA-KO THP-1 cells (Figure 5, D and E). It appeared that the activation of the lectin pathway was enhanced, leading to elevated levels of C3b fragment binding and MAC formation (Figure 5, D and H). The C4d binding on PIGT-KO cells was significantly inhibited by mannosse, suggesting that recognition of mannosse residues in free GPIs by complement lectins might be involved in the lectin pathway activation. It seems likely that enhanced C3 activation is involved in the elevation of serum IL-18 levels because C3 activation continues on PIGT-defective cell-surface during eculizumab therapy. Causal relationship between free GPIs and C3 activation, and the molecular mechanisms involved, needs to be clarified to know whether earlier steps in complement activation are involved in pathogenesis of PIGT-PNH.

Methods

Blood samples and flow cytometry. Peripheral blood samples were obtained from patients J1 (8), G1 (7), G2, and G3 with PIGT-PNH, and 6 patients with PNH after written informed consent. Peripheral blood leukocytes (Supplemental Figure 1B), erythrocytes, and reticulocytes were stained for GPI-APs by FLAER (Cederlane), anti-CD14 (clone MOP9, BD Biosciences), anti-CD16 (clone 3G8, BioLegend), anti-CD24 (clone ML5, BioLegend), anti-CD48 (BJ40, BioLegend), anti-CD59 (clone 5H8) (56), or anti-CD66b (clone 80H3, Beckman Coulter Immunotech). T5-4E10 mAb (T5 mAb) against free GPI of Toxoplasma gondii was a gift from J. F. Dubremetz (18). T5 mAb is now available from BEI Resources, NIAID, NIH (Bethesda, MD). T5 mAb recognizes mammalian free GPI bearing N-acetylgalactosamine (GalNAc) side-chain linked to the first mannose (Figure 3A and ref. 19). T5 mAb does not bind to free GPI when galactose (Gal) is attached to GalNAc. Therefore, reactivity of T5 mAb to free GPI is affected by an expression level of Gal transferase that attaches Gal to the GalNAc. Other antibodies used were PE-anti–CD55/DAF (clone IA10, BD Biosciences), PE-anti-CD88/C5aR (BioLegend), and anti-TR (clone H68.4, Thermo Fisher Scientific). Cells were analyzed by a flow cytometer (MACSQuant Analyzer VYB or FACSCalibur) and FlowJo software.

DNA and RNA analyses. Granulocytes with PNH phenotype were separated from normal granulocytes by cell sorting after staining by FLAER. DNA was analyzed for mutations in genes involved in GPI-AP biosynthesis by target exome sequencing, followed by confirmation by Sanger sequencing (7). DNA was also analyzed by array comparative genomic hybridization for deletion (7). Methylation status of CpG was determined by bisulfite sequencing and a SnuPE assay (13). Total RNA was extracted with the RNeasy Mini Kit (Qiagen) including DNase digestion and DNA cleanup, and reverse transcription was performed with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Levels of L3MBTL1, SGX2, 1FT52, MYBL2, ABL, and GAPDH mRNAs were analyzed by quantitative real-time PCR (Supplemental Table 1).

Cell lines. PIGT-defective CHO cells and PIGL-defective CHO cells were reported previously (11, 57). CRISPR/Cas9 system was used to generate PIGT-KO and PIGA-KO human monocytic THP-1 cells (ATCC) (Supplemental Table 1 for guide RNA sequences). Knockout cells were FACs sorted for GPI-AP-negative cells. Each knockout cell was rescued by transfection of a corresponding cDNA. SLC35A2 gene was knocked out in PIGT-KO THP-1 cells by CRISPR/Cas9 system.

Inflammasome activation and IL-1β measurements. Toll-like receptor 2 (TLR2) ligands PamC54k and Staphylococcus aureus LTA are from InvivoGen (28, 58). ATP and MSU for activating inflammasomes are from Enzo Life Sciences and InvivoGen (28, 59). Peripheral blood mononuclear cells were stimulated by PamC54k or LTA for 4 hours at 37°C and after washing by ATP or MSU for 4 hours at 37°C. IL-1β and IL-18 secretion were measured by ELISA kit (BioLegend) was used to measure IL-1β secreted into the supernatants. Polyclonal rabbit anti–IL-1β antibody for Western blotting was from Cell Signaling Technology. PIGA-KO, PIGT-KO, and WT THP-1 cells were differentiated into adherent macrophages in complete RPMI.
1640 medium containing 100 ng/mL phorbol 12-myristate 13-acetate (PMA; InvivoGen) for 3 hours, and then with fresh complete medium overnight (60). For stimulation, medium was replaced with serum-free medium with PAM, CSK (200 ng/mL), followed 4 hours later by ATP stimulation for 4 hours (5 mM).

Stimulation of THP-1-derived macrophages with complement. As a source of complement, whole blood was collected from healthy donors after written informed consent, and serum separated, aliquoted, and stored at ～80°C prior to use. Inactivation of complement was carried out by heating serum at 56°C for 30 minutes. To prepare AS that allows activation of the alternative pathway on the cell surface, 21 volumes of serum was mixed with 1 volume of 0.4M HCl to have a pH of approximately 6.7. C6- and C7-depleted sera and purified C6 and C7 proteins were purchased from Complement Technology. Differentiated cells were stimulated with acidified normal serum, or acidified C6-depleted and C7-depleted sera, and those reconstituted with C6 and C7, respectively, at 37°C for 5 hours, and secreted IL-1β was measured by ELISA. C5 was inhibited by addition of 35 μg/mL anti-C5 mAb (eculizumab, Alexion Pharmaceuticals).

For ex vivo blockade of human C5aR, anti-human C5aR or nonpeptide C5aR antagonist W-54011 (5 μM, Merck Millipore) (50) was used. Complement C3 and C4 fragments and MAC deposited on the cells were measured by flow cytometry. THP-1 cells were suspended in 20 μL FACS buffer (PBS, 1% BSA, 0.05% sodium azide) with 1:20 human TruStain FcX (Fc receptor blocking solution) at room temperature for 10 minutes. Cells were stained with anti-C3/C3b/iC3b/C3d mAb (clone 1H8, BioLegend), anti-C4d mAb (clone 12D11, Hycult Biotech) or rabbit anti-human SC5b-9 (MAC) polyclonal antibodies (Complement Technology) in FACS buffer. After washing twice, cells were incubated with the PE-conjugated goat anti-mouse IgG (BioLegend) or Alexa Fluor488-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific) secondary antibody. The anti-human SC5b-9 polyclonal antibodies positively stained PMA-differentiated THP-1 cells without incubation in AS. The same antibodies did not stain similarly differentiated PIGT-KO and PIGA-KO THP-1 cells, suggesting that the antibody product contained antibodies reacted with some GPI-AP expressed on THP-1–derived macrophages (Supplemental Figure 8, B and C). Because of this reactivity to non-MAC antigen(s), the anti-SC5b-9 antibodies were used for PIGT-KO and PIGA-KO cells but not for WT cells in experiments shown in Figure 5, D and E. To inhibit the lectin pathway, serum was mixed with mannose or N-acetylglucosamine (final concentration of 100 mM) before acidification (61).

Statistical analyses. All experiments with THP-1 cells were performed at least 3 times. All values were expressed as the mean ± SD of individual samples. For 2-group comparisons between PIGT-KO and PIGA-KO cells, 2-tailed Student’s t test was used. P values less than 0.05 were considered statistically significant.

Study approval. This study was approved by the institutional review boards of Osaka University (approval number 681), University of Ulm (approval numbers 279/09 and 188/16), and University of Berlin (approval number EA2/077/12).

Data sharing statement. All data supporting the findings are available from the corresponding authors.

Author contributions

BH, YM, NI, HS, PMK, and TK designed research. YM, MO, AK, TH, S Murata, TE, MJ, RF, and AH performed research. BH, MK, MA, S Murase, YU, and NK acquired the data. BH, YM, MO, MK, JN, YK, NK, HS, and PMK analyzed data. BH, YM, MO, HS, PMK, and TK wrote the paper. Three authors sharing the first author position are in alphabetical order.

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